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Breast cancer cells that metastasize to the bone marrow early in the course of the disease can become dormant and are resistant to the chemotherapy given to eliminate them. We developed a model of dormancy in vitro where well-differentiated breast cancer cells incubated with basic fibroblast growth factor (FGF-2) are growth-inhibited and upregulate integrins through which they receive survival signaling. Interaction between integrin alpha 5 beta 1 and fibronectin provides a survival advantage to dormant clones. Dormant cells have activated Akt and are resistant to docetaxel. Blocking Akt and PI3 kinase, its upstream activator, using specific inhibitors reverses the survival advantage conferred by fibronectin but does not abrogate dormant clones completely. Blocking PI3 kinase has the additional effect of disrupting the morphologic characteristics of dormant cells, suggesting a role for additional pathways besides Akt downstream of PI3 kinase in dormant cell survival. A transcription inhibitor, flavopiridol, was very effective in diminishing survival of dormant clones. While it inhibited Akt phosphorylation, virally transduced replacement of Akt function did not reverse the inhibitory effects of flavopiridol. Flavopiridol reversed the upregulation of integrins and diminished adherence, particularly to fibronectin, supporting a role for this mechanism for its effects on dormant cells.

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INTRODUCTION

Objectives/Hypothesis: Microscopic metastases found in the bone marrow of patients diagnosed with primary breast cancer are resistant to chemotherapy. The bone marrow microenvironment can provide growth inhibition and protection from cell death to many cell types through interactions between stromal proteins and cell surface receptors called integrins. Growth factors in the bone marrow can influence the expression of these receptors, making the cell express specific integrin combinations that are best suited for interactions between the proteins present in the marrow and the target cell. These interactions can initiate survival signaling that result in dormancy and resistance to chemotherapy.

Our data suggests that basic fibroblast growth factor (FGF-2), a peptide growth factor present in abundance in the bone marrow, induces metastatic breast cancer cells to stop proliferating and change their integrin expression pattern to include and overexpression of integrins $\alpha 5$ and $\beta 1$. Without appropriate adherence and specific ligation, this integrin induces massive cell losses. A specific ligand for integrin $\alpha 5 \beta 1$ present in the bone marrow, fibronectin, binds to the cells through this integrin and initiates survival signaling. In an *in vitro* model of dormancy, we have demonstrated that breast cancer cells have increased Akt phosphorylation in the presence of FGF-2. FGF-2 induced the phosphorylation of Akt in MCF-7 cells on fibronectin. The effect was strongest after 24 hours but remained sustained for the 5 days assayed. FGF-2 also induced sustained Akt phosphorylation in T-47D cells. GSK-3 α and β , downstream substrates of Akt were phosphorylated on Ser 21/9 in both dormant MCF-7 and T-47D cells on fibronectin on day 6, as determined by Western blots, indicating that phosphorylation of Akt resulted in its activation. In MDA-MB-231 cells that do not undergo dormancy, Akt was phosphorylated at baseline on fibronectin and did not respond to FGF-2 treatment. Studies presented here demonstrate the role of the activation of Akt and its upstream activator, PI3 kinase in survival of dormant clones. They also demonstrate the efficacy of a transcription inhibitor, flavopiridol, in inhibiting the survival of dormant clones resistant to a taxane, a classical chemotherapeutic agent used in the treatment of breast cancer. Data will demonstrate potential mechanisms of flavopiridol on eliminating dormant clones, through decreased expression of integrins and down-modulation of adherence.

Study design: Experiments were carried out with MCF-7 and T-47D cells, two well differentiated cell lines that are inhibited from proliferating and induced into a less malignant state by FGF-2, as our previous studies demonstrated (1-2). Cells were incubated in tissue culture dishes or dishes coated with fibronectin in the presence or absence of 10 ng/ml FGF-2 at clonogenic densities of 1,000 or 2,000 cells per well of 24 well plates. As a control, we used MDA-MB-231 cells that are highly de-differentiated and are not growth inhibited by FGF-2. The various inhibitors are added on day three of incubation and the cells are fixed and stained on day 6 of incubation (3). Dormant clones of 2-10 cells and growing clones of >29 cells were counted. Proliferation assays and western blots were carried out as before (1-3). For western blots, cells were incubated on fibronectin-coated plates at low density to minimize cell-cell contact.

BODY

A. Determine the significance of the PI3 kinase/Akt pathway in survival of dormant clones.

To determine the significance of Akt phosphorylation in dormancy, the effects of the specific chemical Akt inhibitor I (Calbiochem) was investigated in the clonogenic assays. Addition of the inhibitor on day three resulted in a dose-dependent decrease of dormant clones on day 6, with an

ED₅₀ of 5 μ M, the reported K_d of the compound (Figure 1). The inhibitor was slightly more effective in T-47D cells than in MCF-7 cells, but did not eradicate dormant clones much past the level supported on plastic in either cell line, essentially reversing the survival effect afforded by fibronectin. The inhibitor did not have a significant effect on growing clones, a condition where Akt was not phosphorylated.

Figure 1. Inhibition of activated Akt restricts dormant clone survival on fibronectin.

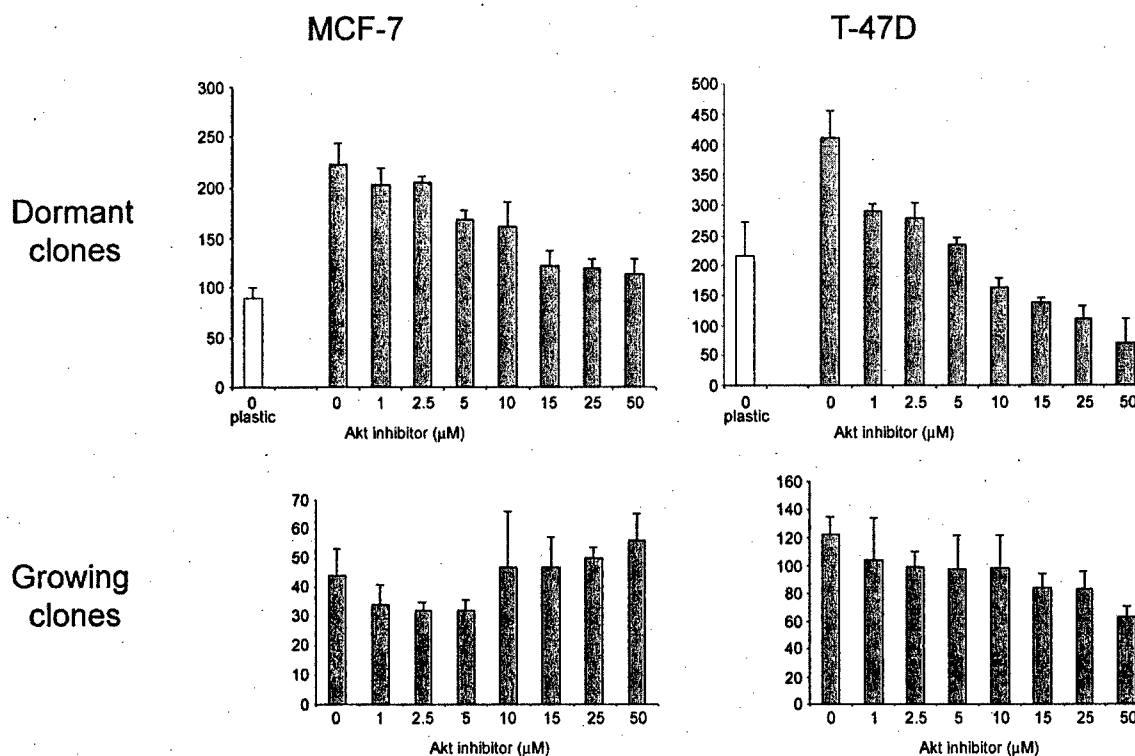


Figure 1 MCF-7 and T-47D cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of Akt inhibitor were added after 3 days. Cells were stained with crystal violet at 6 days and colonies with ≤ 10 cells in FGF-2-treated plates (dormant clones) and with ≥ 29 cells on cultures lacking FGF-2 (growing clones) were counted. Colonies on tissue culture plates are shown as baseline control for dormant clones. Error bars, \pm S.D.

Inhibition of PI3K, the upstream activator of Akt, with LY294002 also inhibited dormant clone survival in a dose-dependent manner, with an ED₅₀ of approximately 10 μ M (Figure 2A). However, dormant clones approximating in number those sustained on plastic were not eradicated by inhibition of PI3K either. In contrast to Akt inhibition, however, LY294002 inhibited proliferating clones in a dose dependent manner with an ED₅₀ of about 5 μ M, supporting the role of PI3K in their survival. The difference in blocking PI3K and its downstream target Akt was further demonstrated by the appearance of the surviving dormant clones on fibronectin (Figure 2B). While dormant cells surviving Akt inhibition remained characteristically large, spread out with large cytoplasm to nucleus ratios, surviving cells after LY294002 treatment were small, dendritic and appeared distressed. This suggested that additional pathways, other than Akt, downstream of PI3K may affect the cytoskeletal changes of dormancy. These are currently under investigation. As a control, LY294002 also inhibited the growing MDA-MB-231 clones equally with and without FGF-2 treatment but required a concentration about ten times higher than that needed to inhibit growing MCF-7 or T-47D clones (Figure 2C).

Figure 2. Inhibition of PI3K restricts dormant clone survival on fibronectin.

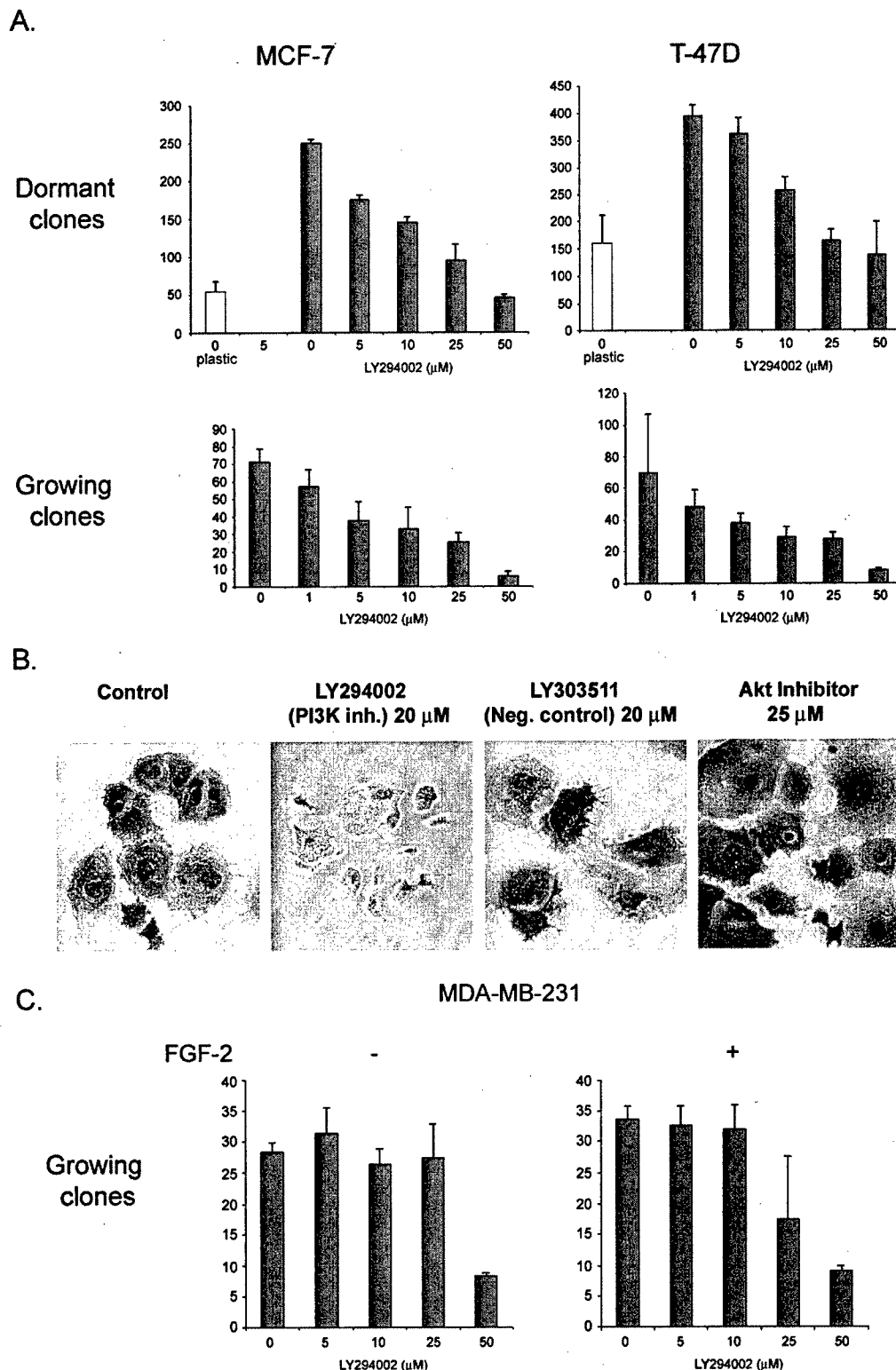


Figure 2 A. MCF-7 and T-47D cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of LY294002 were added after 3 days. Cells were stained with crystal violet at 6 days and colonies with ≤ 10 cells in FGF-2-treated plates (dormant clones) and with ≥ 29 cells on cultures lacking FGF-2 (growing clones) were counted. LY303511 50 μ M was used as a negative control (not shown). Colonies on tissue culture plates are shown as baseline control for dormant clones. **B.** Morphogenic appearance of dormant T-47D cells on fibronectin treated for 3 days with Akt inhibitor, LY294002 and LY303511 on day 6 of culture. **C.** MDA-MB-231 cells were incubated with or without FGF-2 on fibronectin-coated plates. Variable concentrations of LY294002 were added after 3 days. Cells were stained with crystal violet at 6 days and colonies with ≥ 29 cells were counted (there were no dormant clones evident). Error bars, \pm S.D.

B. A role for flavopiridol in inhibiting survival of dormant breast cancer cells

To determine if our paradigm reproduced the *in vivo* chemoresistance of dormant cells in the bone marrow, we determined the susceptibility of dormant breast cancer cells to the chemotherapeutic agent docetaxel relative to that of growing cells. We used the two well-differentiated breast cancer cell lines in our ongoing dormancy study, analogous to cells that are typically capable of remaining dormant as micrometastases in the bone marrow of patients with breast cancer (4, 5) and that formed dormant clones in our *in vitro* model (3). In a clonogenic assay on fibronectin, the ED_{50} of docetaxel was also under 1 nM, with 10 nM completely obliterating the survival of any growing clones (Figure 3A). In contrast, docetaxel concentrations as high as 100 nM were not capable of eliminating the dormant cell clones cultured on fibronectin (Figure 3B). Similar data were obtained with MCF-7 cells (data not shown).

Figure 3. Docetaxel inhibits survival of growing but not of dormant breast cancer cells.

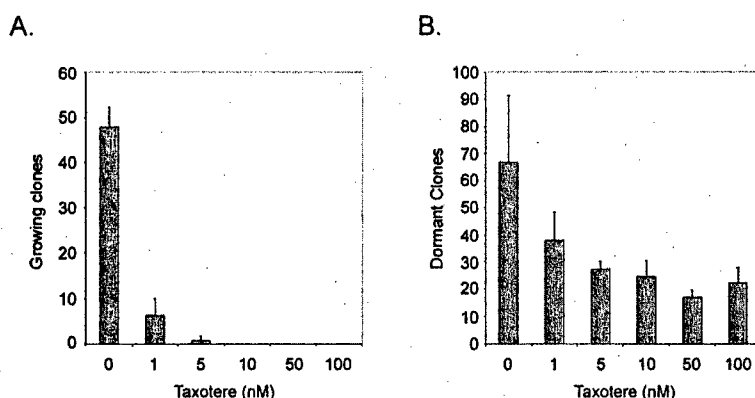


Figure 3. Clonogenic assay of T-47D cells on fibronectin without (A.) and with (B.) 10 ng/ml FGF-2 incubated with variable concentrations of docetaxel. The cells were incubated with and without FGF-2 at 1,000 cells/well in 24 well fibronectin-coated plates and the media and FGF-2 were changed and docetaxel was added after three days in culture. Cells were fixed and stained on day 6 and growing and dormant colonies were counted as described in Study Design.

Our studies have attributed specific interactions between integrins $\alpha 5$ and $\beta 1$ and fibronectin to survival effects in dormant clones. In this model, FGF-2, a growth factor abundant in the bone marrow stroma, inhibited the proliferation of well-differentiated breast cancer cells and established clones that were growth-arrested at a few cell stage that we termed dormant (8). FGF-2 induced an upregulated expression of the mRNA and protein levels of a number of cellular integrins, including integrins $\alpha 5$ and $\beta 1$, a pair that specifically provided a survival advantage to these dormant clones when cultured on a fibronectin substratum (8). We hypothesized that flavopiridol, a transcription inhibitor, would interfere with the upregulated expression of these integrins and inhibit the survival advantage provided by their ligation. Figure 4 demonstrates that flavopiridol did indeed inhibit the upregulated expression of integrins $\alpha 5$ and $\beta 1$, as well as $\alpha 2$, $\beta 3$ and $\beta 4$, the other integrins that were induced by FGF-2 in MCF-7 and T-47D cells. The effect became evident at 100 nM to 150 nM flavopiridol.

Figure 4. Flavopiridol inhibits expression of integrins $\alpha 2$, $\alpha 5$, $\beta 1$, $\beta 3$ and $\beta 4$ upregulated by FGF-2 on fibronectin.

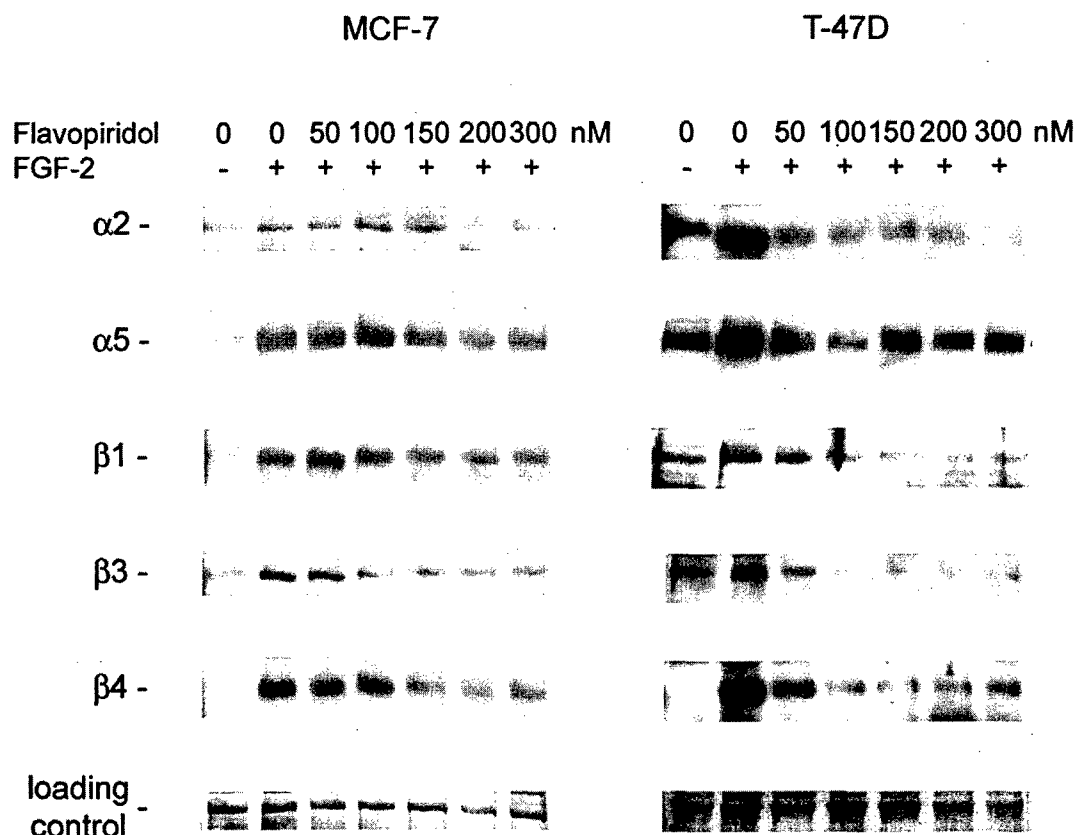


Figure 4. T-47D cells were incubated at low density on fibronectin-coated tissue culture plates in standard media. The following day, the media was replaced with fresh media with and without FGF-2. One day later, flavopiridol was added at the variable concentrations indicated along with fresh media and FGF-2. Lysates were prepared 48 hours later and analyzed by western blot with antibodies to the integrins shown. Nonspecific bands from Coomassie blue-stained membranes were used to verify equal loading.

Based on these results, we hypothesized that flavopiridol would inhibit the survival of dormant clones. We determined the effects of flavopiridol on dormant clones and compared the effects with those obtained on growing cells. Figure 5A demonstrates that flavopiridol inhibited the proliferation of T-47D cells on tissue culture-coated plates in a dose dependent manner with an ED_{50} of 25 nM. Similarly, the ED_{50} for inhibition of growing clones on fibronectin was less than 50 nM (Figure 5B). In contrast to the effects observed with docetaxel, flavopiridol almost completely eradicated dormant T-47D clones (Figure 5C). The ED_{50} was between 50 and 100 nM, with almost complete elimination of dormant clones occurring at 200 nM. Similar results were observed with MCF-7 cells (data not shown).

We investigated potential mechanisms for the eradication of dormant clones by flavopiridol. We previously demonstrated that FGF-2 induced a sustained increase in the phosphorylation of Akt in dormant cells that conferred a specifically reversible protection to dormant cells attached to fibronectin (3). To determine if flavopiridol-induced inhibition of dormant colony survival is mediated through interference with Akt phosphorylation we performed western blots with anti-phospho-Akt antibody on lysates from dormant cells treated with variable doses of flavopiridol. Figure 6 demonstrates that flavopiridol decreased the Serine 473 phosphorylation of Akt after 24 hours when added to cells in which dormancy was established on fibronectin after a three day incubation with FGF-2. The inhibition was evident at 100 to 150 nM flavopiridol. Total Akt levels while slightly diminished but remained abundant. The dephosphorylation of Akt was accompanied by a dose-dependent dephosphorylation of Ser21/9 of GSK3 α/β , demonstrating a functional significance to the dephosphorylation of Akt.

Figure 5. Flavopiridol inhibits survival of growing and dormant breast cancer cells.

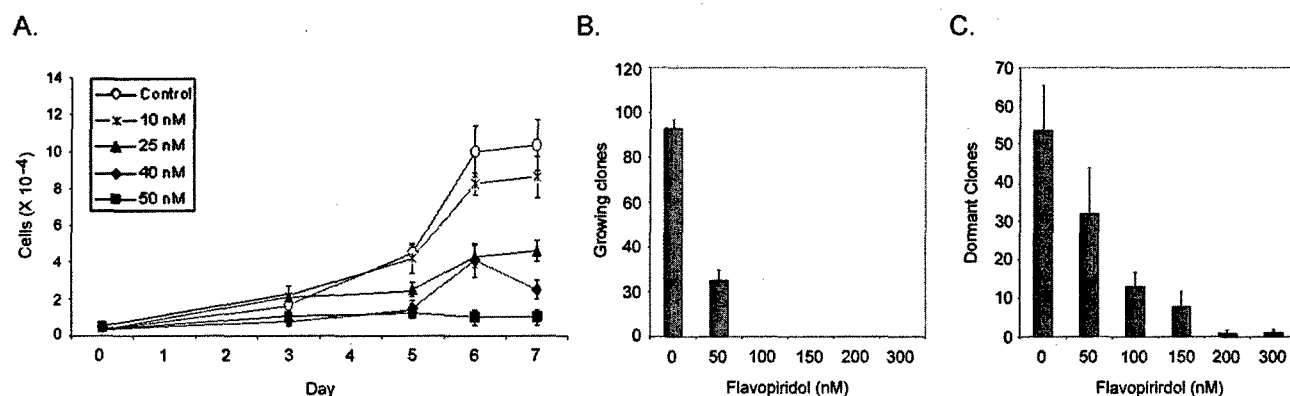


Figure 5. A. Proliferation assay of T-47D cells incubated on tissue culture-coated plates with variable concentrations of flavopiridol for the times indicated and counted in 0.2% trypan blue. Clonogenic assay of T-47D cells on fibronectin without (B.) and with (C.) 10 ng/ml FGF-2 incubated with variable concentrations of flavopiridol. Cells were incubated with and without FGF-2 at 1,000 cells/well in 24 well fibronectin-coated plates, the media and FGF-2 were replaced and flavopiridol was added after three days. Cells were fixed and stained on day 6. Growing and dormant clones were counted as described above.

Figure 6. Flavopiridol inhibits Akt activation induced by FGF-2 on fibronectin

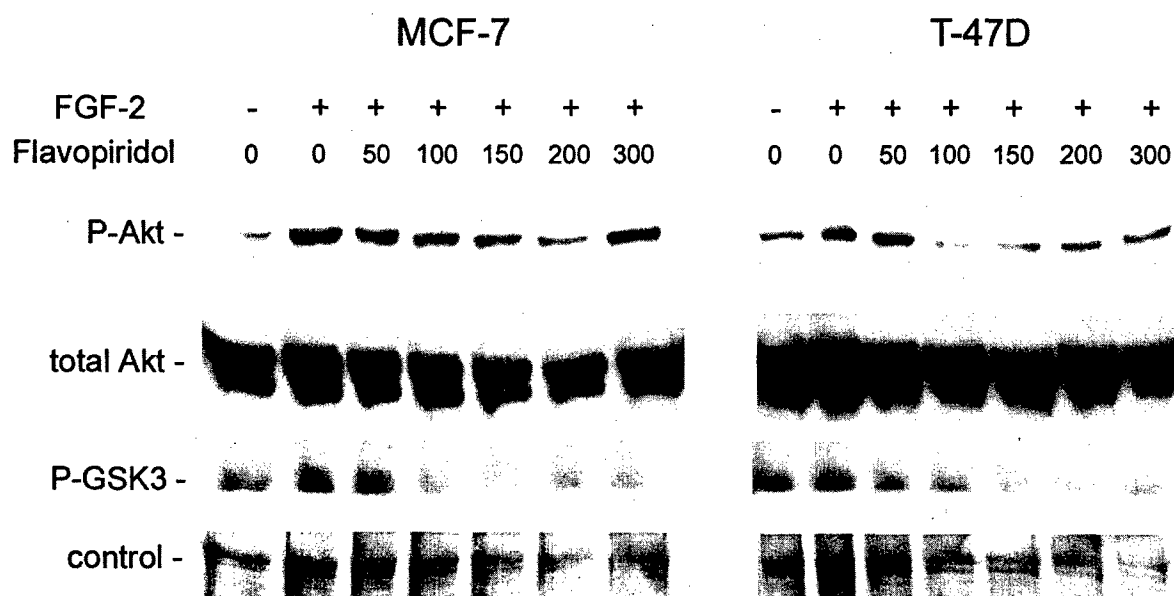


Figure 6. Cells were incubated on fibronectin-coated plates and fresh media with and without 10 ng/ml FGF-2 was added the following day. Flavopiridol was added on day 2, lysates were prepared on day 4 and analyzed by Western with A. anti phospho-Akt and total Akt antibodies and B. with antibody to phosphorylated Ser21/9 of GSK-3alpha/beta isoforms. Non-specific bands on Coomassie blue-stained gels were used to determine loading.

We wanted to determine if the inhibition of Akt phosphorylation was a primary mechanism for flavopiridol-induced eradication of dormant clones. We transduced the cells with adenoviral vectors and a control vector expressing β -gal to determine if a constitutively active Akt mutant can reverse dormant clone inhibition by flavopiridol. Adenoviral vectors expressing His-tagged wild type Akt, a dominant negative Akt mutant and a constitutively active Akt mutant or a β -gal gene were constructed, cultured and titered as described (6). Dormant cells incubated for three days with

FGF-2 10 ng/ml on fibronectin were infected with adenoviral supernatant on day three at variable multiplicities of infection (MOI's). Transduction efficiencies were in the 30% range at MOI's from 5 to 50 as determined by transductions with the β -gal vector and immunohistochemical detection (data not shown). Lysates were collected 24 and 72 hours after addition of viral supernatants. Figure 7 is a western blot stained with antibody to His demonstrating expression of His-tagged Akt isoforms in MCF-7 cells transduced with the adenoviral vectors at MOIs of 2 and 5, and the resultant inhibition of GSK phosphorylation by expression of dominant negative Akt 72 hours after transduction. Similar results were obtained with T-47D cells (data not shown). To determine the contribution of Akt inactivation on flavopiridol-induced dormant clone survival, MCF-7 and T-47D cells were incubated with FGF-2 10 ng/ml on fibronectin and transduced two days later with the constitutively active Akt adenoviral vectors at variable MOI's up to 50. Wild type and dominant negative Akt and β -gal vectors were used as controls. On day 3, the media was changed, and included FGF-2 10 ng/ml, flavopiridol 100 nM and viral supernatants. Colonies were stained on day 6 and counted. Flavopiridol consistently inhibited clonal survival, as before. However, neither constitutively active nor wild type Akt transduction had any significant effects on reversing this inhibition (data not shown). This suggested that while flavopiridol reversed the activation of Akt signaling in dormant clones, this effect was not the only nor the primary mechanism of flavopiridol-mediated abrogation of dormant clone survival in these well-differentiated breast cancer cells.

Figure 7. Enforced expression of adenoviral mediated Akt mutants in MCF-7 cells.

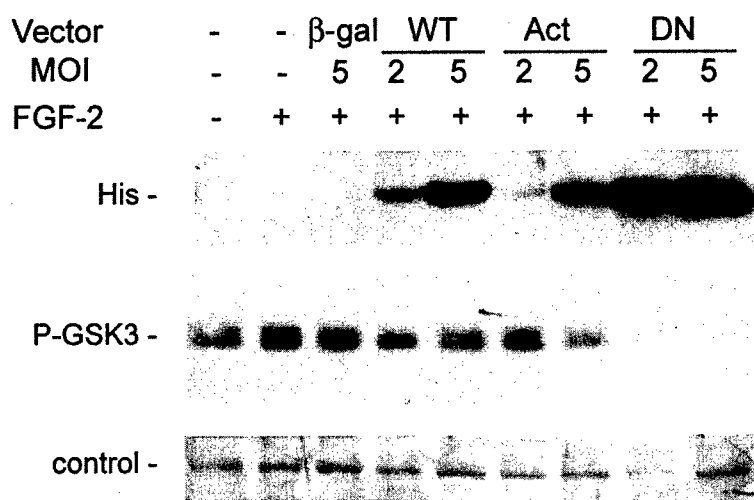


Figure 7. Cells were incubated with FGF-2 on fibronectin-coated plates. On day 3, fresh media was added containing FGF-2 and adenoviral vectors at the MOI's shown. Lysates were collected 24 and 72 hours later. Shown is the western blot for His and phospho-GSK3 at 72 hours. Non-specific bands on Coomassie blue-stained gels were used to determine loading.

We have previously demonstrated that blocking the interaction of integrins α 5 and β 1 and fibronectin with specific blocking peptides and blocking antibodies reversed the survival advantage provided to dormant clones by fibronectin ligation (3). Because flavopiridol decreased the levels of expression of these integrins, we investigated whether the effect resulted in a decrease in adhesion to fibronectin as a potential mechanism for the effects of flavopiridol on survival of dormant clones. We incubated cells on fibronectin-coated plates, and on collagen I- and tissue culture-coated plates as controls, for three days with and without FGF-2 10 ng/ml. On day 3, flavopiridol 200 nM was added and on day 4, susceptible cells were de-attached from the plates using Cell Dissociation Media. The cells that remained in the plate were fixed and stained with

Figure 8. Flavopiridol reverses FGF-2-induced cell adhesion in dormant cells.

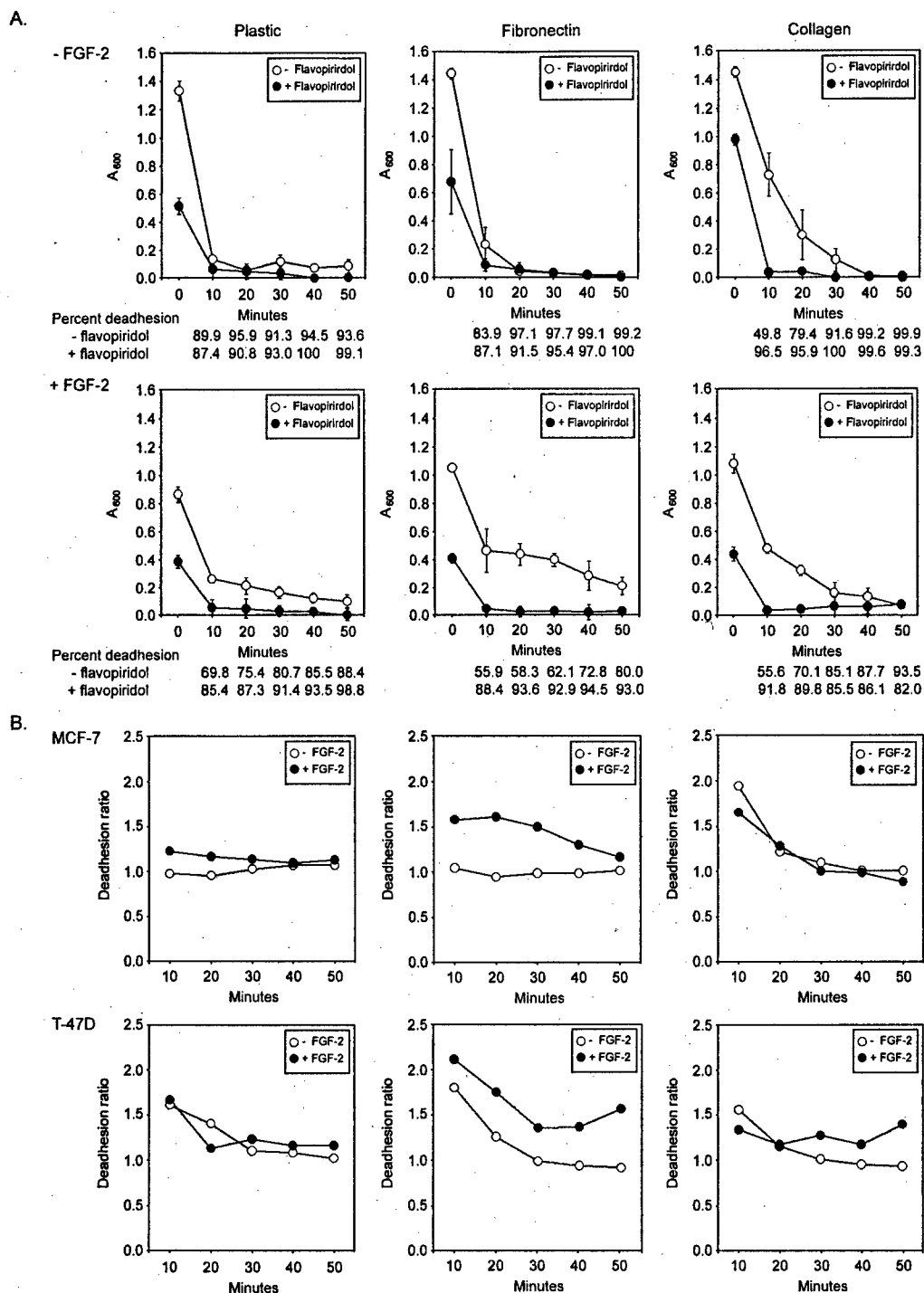


Figure 8. Cells were cultured on tissue culture-coated (plastic), fibronectin-coated or collagen I-coated 24 well plates at 20,000 MCF-7 cells/well(15,000 T-47D cells/well) \pm FGF-2 10 ng/ml for 3 days. The media and FGF-2 were replaced on day 3 and flavopiridol 200 nM was added. On day 4, cells were washed and incubated with 0.25 ml Cell Dissociation Solution (Sigma, St. Louis, MO) at 37°C 5% CO₂ for variable times, washed once with PBS and stained with 0.1% crystal violet solution, as described. The dye was extracted with 10% acetic acid and the A₆₀₀ was measured. Background absorbance obtained from wells with no cells remaining, usually at 50 minutes for each substratum type without FGF-2, was subtracted from absorbance values for graphing. A. One typical experiment with MCF-7 cells demonstrating differential effects of flavopiridol on growing compared to dormant, FGF-2-treated cells. Percent de-adhesion was calculated as $1 - A_{600}/A_{600}t_0$ where t is the time of incubation with Cell Dissociation Solution and t_0 is the 0 time point without de-adhesion and A_{600} is the corrected absorbance. Error bars are \pm S.D. B. Accentuated difference in the deadhesion ratio (percent deadhesion + flavopiridol/percent de-adhesion - flavopiridol) between FGF-2 treated and rapidly growing cells in fibronectin compared to plastic or collagen I in the experiment shown in A with MCF-7 cells and a comparable experiment with T-47D cells. All experiments were done in quadruplicate and carried out at least twice.

crystal violet solution, extracted with 10% acetic acid and quantitated by absorbance at A_{600} . Figure 8 demonstrates the differential effects of flavopiridol on growing compared to dormant FGF-2-treated cells in a typical experiment with MCF-7 cells. Deadhesion in the absence of FGF-2 was rapid and nearly total on plastic and fibronectin. On collagen, deadhesion was also near total but followed slower kinetics. Upon incubation with FGF-2, cells became only slightly more adherent to plastic but their adherence to fibronectin increased markedly both with MCF-7 cells (Figure 8A) and T-47D cells (not shown). Their adherence to collagen, however, was not affected by FGF-2 treatment. The tables below the graphs indicate the percent deadhesion calculated as $1 - A_{600t}/A_{600t_0}$ where t is the time of incubation with Cell Dissociation Solution, t_0 is the 0 time point without de-adhesion and A_{600} is the absorbance corrected by subtracting the background from the stained wells containing no cells. When the cells were incubated with 200 nM flavopiridol for 24 hours prior to deadhesion, however, the cells on all of the substrata with and without FGF-2 treatment deadhered quickly and completely. Thus, flavopiridol reversed the fibronectin-specific increase in adherence in cells incubated with FGF-2. To demonstrate the fibronectin specific effect, we plotted the deadhesion ratio (percent deadhesion, + flavopiridol/percent de-adhesion, no flavopiridol) as a function of time, comparing FGF-2 treated cells and rapidly growing cells on the three substrata for the experiment shown in Figure 8A for MCF-7 cells and a comparable experiment with T-47D cells (Figure 8B). The deadhesion ratios on plastic and collagen were similar or overlapping for FGF-2 treated or untreated cells on plastic and collagen for both cell lines but they diverged widely and consistently for the entire time course on fibronectin, again supporting the effect of flavopiridol in reversing a specific adherence in dormant cells endowed by incubation with FGF-2. These experiments support a potential mechanism for flavopiridol in eliminating survival of dormant clones on fibronectin by disrupting their specific interaction with the supporting substratum.

KEY RESEARCH ACCOMPLISHMENTS

1. Signaling through Akt contributes to survival of dormant breast cancer cells.
2. Signaling through PI3 kinase contributes to survival of dormant breast cancer cells and contributes to morphologic characteristics of dormant cells through pathways other than Akt.
3. Dormant cells are resistant to docetaxel.
4. Flavopiridol inhibits survival of dormant clones.
5. Flavopiridol downregulates signaling through Akt but this effect is not the mechanism responsible for flavopiridol-mediated inhibition of dormant clones.
6. Flavopiridol reverses the increased expression of integrins associated with dormancy.
7. Flavopiridol preferentially decreases adhesion to fibronectin supporting this as a mechanism for its elimination of dormant clones.

REPORTABLE OUTCOMES

Korah R, Boots M, and Wieder R. (2004) Integrin $\alpha 5\beta 1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. *Cancer Research* 64: 4514-4522.

Saltanat Najmi, Vineetha Joseph, Monika Boots, Reju Korah and Robert Wieder Flavopiridol blocks integrin-mediated survival signaling in dormant breast cancer cells. (2003) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 69, #P62.

Robert Wieder, Monika Boots, and Reju Korah. Integrin $\alpha 5\beta 1$ promotes survival of growth-arrested breast cancer cells: an *in vitro* paradigm for breast cancer dormancy in bone marrow. (2003) Annual Retreat on Cancer Research in NJ, The Cancer Institute of NJ and the NJ State Commission on Cancer Research, p. 33, #E2 (Selected for oral presentation).

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CONCLUSIONS

1. Survival of dormant clones is mediated at least in part through the PI3 kinase/Akt pathway, although additional pathways downstream of PI3 kinase may play a role. Blocking signaling through either kinase alone does not eliminate dormant clones completely but eliminates the incremental effect on survival provided by incubation on fibronectin.
2. Dormant clones are resistant to docetaxel chemotherapy.
3. Flavopiridol inhibits survival of dormant clones. While it inhibits Akt-mediated signaling, this is not likely the primary mechanism. Flavopiridol also reverses the increased expression of integrins acquired with dormancy and this effect results in a diminished adhesive capacity, in particular to fibronectin. This provides a likely mechanism for the effects of flavopiridol on dormant breast cancer clones.

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